Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata* L.) to environmental stress: New insights in fish mitochondrial phenotyping

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Abstract

The aim of the current study was to phenotype fish metabolism and the transcriptionally-mediated response of hepatic mitochondria of gilthead sea bream to intermittent and repetitive environmental stressors: i) changes in water temperature (T-ST), ii) changes in water level and chasing (C-ST) and iii) multiple sensory perception stressors (M-ST). Gene expression profiling was done using a quantitative PCR array of 60 mitochondria-related genes, selected as markers of transcriptional regulation, oxidative metabolism, respiration uncoupling, antioxidant defense, protein import/folding/assembly, and mitochondrial dynamics and apoptosis. The mitochondrial phenotype mirrored changes in fish performance, haematology and lactate production. T-ST especially up-regulated transcriptional factors (PGC1 α , NRF1, NRF2), rate limiting enzymes of fatty acid β -oxidation (CPT1A) and tricarboxylic acid cycle (CS), membrane translocases (Tim/TOM complex) and molecular chaperones (mtHsp10, mtHsp60, mtHsp70) to improve the oxidative capacity in a milieu of a reduced feed intake and impaired haematology. The lack of mitochondrial response, increased production of lactate and negligible effects on growth performance in C-ST fish were mostly considered as a switch from aerobic to anaerobic metabolism. A strong down-regulation of PGC1 α , NRF1, NRF2, CPT1A, CS and markers of mitochondrial dynamics and apoptosis (BAX, BCLX, MFN2, MIRO2) occurred in M-ST fish in association with the greatest circulating cortisol concentration and a reduced lactate production and feed efficiency, which represents a metabolic condition with the highest allostatic load score. These findings evidence a high mitochondrial plasticity against stress stimuli, providing new insights to define the threshold level of stress condition in fish.

Keywords: husbandry stress; mitochondrial metabolism; teleost; thermal stress.

1. Introduction

Mitochondria are cellular organelles that play a variety of important roles in eukaryotic cell physiology, ranging from production of ATP and redox homeostasis to biosynthesis of macromolecules and intracellular calcium regulation, which are related to different pathways that influence cellular homeostasis and fate, including cell death cascades (Galluzzi et al., 2012). Dysfunction of this cell organelle is, thereby, associated with the natural chronic process of ageing, as well as with neurodegenerative disorders, metabolic diseases and toxic insults (Scharfe et al., 2009). The number of mitochondria and their level of activity also vary depending on tissue and cell type, reflecting the energy requirements of the cell. Both can be modulated by internal and external factors through the tight transcriptional and translational regulation of nuclear and mitochondrial proteins (Bolender et al., 2008; Garesse and Vallejo, 2001; Scheffler, 2001). This includes induction of protein transcriptional co-activators, import of precursor proteins into mitochondria, as well as incorporation of both mitochondrial and nuclear gene products into the expanding organelle reticulum. Each of these steps adapts to altered physiological conditions in order to regulate cellular homeostasis, and recent reviews in humans and other animal models have summarized the current knowledge on most of these processes. Thus, mitochondria biogenesis can be activated by physiological and pathological stimuli, such as exercise, caloric restriction, thermogenesis, postnatal breathing, secretion of thyroid hormone and erythropoietin, oxidative stress and inflammation (Chen et al., 2009; Ljubacic et al., 2010; Piantadosi and Suliman, 2012a, b).

Literature on the regulation of mitochondrial activity and biogenesis is poorer in fish than in humans and higher vertebrates, although it appears that fish mitochondria are especially versatile (O'Brien, 2011). Hence, fish mitochondrial activity is highly

modulated by thermal (Beck and Fuller, 2012; Egginton and Johnston, 1984; Guderley, 1997; Mueller et al., 2011; Orczewska et al., 2010), osmotic (Tse et al., 2012), chemical (Peter et al., 2013) or nutritional stressors (Enyu and Shu-Chien, 2011). In particular, mitochondrial function in gilthead sea bream (*Sparus aurata*) is highly regulated by dietary oils (Pérez-Sánchez et al., 2013), but it remains largely unclear how different stressors induce mitochondrial damage, energy failure and cell death, and more importantly, how these processes initiate retrograde signals for transcriptional regulation of mitochondrial biogenesis and cell-tissue repair. Furthermore, there is not a consensus endocrine profile for chronically stressed animals or how to assess it without invoking further stress (Dickens and Romero, 2013; Pankhurst, 2011). This notion is extensive to gilthead sea bream exposed to chronic and acute stress (Arends et al., 1999; Caldach-Giner et al., 2010; Fanouraki et al., 2011; Rotllant et al., 2000), but even in a higher extent when the less studied intermittent and repetitive stressors are considered (Ibarz et al., 2007; Tort et al., 2001). These type of stressors typically include daily farming activities, such as people walking alongside tanks and removal of dead fish, as well as activities that involve changes in noise and/or light level, potentially giving rise to a wide variety of stimuli that most fish adapt to slowly and are difficult to quantify (Bratland et al., 2010; Nilsson et al., 2012).

The current methodological constraints can be partially overcome with the advent of improved genomic resources for the most important cultured fish species. This is the case of gilthead sea bream (Caldach-Giner et al., 2013), for which an updated reference transcriptome database with a high representation of mitochondrial-related transcripts is now available at www.nutrigroup-iats.org/seabreamdb. This has allowed the development and validation of a mitochondrial quantitative PCR array that profiles the expression of 60 genes, selected as markers of nuclear transcriptional regulation (5

genes), oxidative metabolism/respiration uncoupling (13 genes), antioxidant defense (7 genes), protein import/folding/assembly (23 genes), and mitochondrial dynamics and apoptosis (12 genes). These markers were selected on the basis of the transcriptionally-mediated responses of gilthead sea bream to crowding stress (Bermejo-Nogales et al., 2008; Calduch-Giner et al., 2010; Saera-Vila et al., 2009), and literature references in other animal models, including rodents and humans (Liesa et al., 2009; Ljubicic et al., 2010; Manoli et al., 2007; Wenz, 2013). This molecular phenotyping was then completed with measurements of haematological parameters, plasma hormones and metabolites, including cortisol, glucose and lactate as a marker of anaerobic metabolism. The final aim was to determine whether mitochondrial response could be used as a highly integrative and informative tool capable of phenotyping stress in fish, providing at the same time new tools and insights to define the threshold level of stress condition in cultured fish.

2. Materials and methods

2.1 Experimental setup

Juvenile gilthead sea bream of Atlantic origin (Ferme Marine de Douhet, France) were acclimatized to the indoor experimental facilities of the Institute of Marine Research (IMR), Matre Research Station (Norway) for two months. Fish (265–274 g average body weight) were then distributed into twelve 500L tanks (27 fish per tank) at a stocking density of 14–15 kg/m³. Each tank was closed with a lid fitted with two fluorescent light tubes (18 Watt each) and one automatic feeder (RVO-TEC T Drum 2000, Arvotec, Huutokoski, Finland). A 12D:12L photoperiod was maintained with lights on from 8:00 h to 20:00 h. All tanks were supplied with heated seawater (salinity 35‰) that was maintained at 20°C with a flow rate of 24–32 L/min. Fish were fed 4.5

mm dry pellets (EFICO YM 554, BioMar, Dueñas, Palencia, Spain) twice a day (11:00 h and 16:00 h) near to satiation 7 days per week. Feed intake was collectively and daily monitored for each tank (experimental unit) through all the stress trial. Three weeks prior to the start of the stress trial, feed intake was also checked in order to ensure that there were no major tank effects in the trial.

Four groups, corresponding to control (CTRL) fish and three groups of stressed (ST) fish, were established in triplicate for an experimental period of 21 days. Fish assigned to the thermal stressed group (T-ST) were under water temperature cycles of 2 days at 12°C to 3 days at 20°C. Regulation of water temperature was done manually in the morning (start time 9:00 h), lasting approximately 4 h. In the chasing stress group (C-ST), water level in the tank was lowered twice a day (9:15 h and 14:15 h) to 10 cm and was kept at this level for 45 min. Thirty min after lowering water level, fish were intensively chased with a pole for 5 min. Fish assigned to the multiple sensory perception stressors group (M-ST) were under a fast series of automated stressors for 30 min three times a day (9:30h, 14:30 h and 18:30 h). During the stress time, fish were exposed to a short burst (10 sec) of four different stressors in a random order: i) a massage device shook the tanks and made a sound, ii) a window wiper moved back and forth in the water, iii) a pump reversed the water flow and iv) a strobe light caused flashes of light.

At the end of the experiment, 6 fish per tank (18 fish in total per experimental condition) were randomly sampled and anaesthetized in a bucket containing 0.1 g/L of 3-aminobenzoic acid ethyl ester (MS-222; Sigma, Saint Louis, MO, USA). Blood was quickly drawn from caudal vessels. The total time including anaesthesia and blood withdrawal was 4 min for all sampled fish in a given tank. One aliquot of blood was used for haematocrit and haemoglobin measurements. Remaining blood was centrifuged

at 3000 g for 20 min at 4°C, and plasma samples were frozen and stored at -20°C until cortisol and metabolite analyses. Prior to tissue collection, fish were killed by cervical section. The liver was then rapidly harvested, frozen in liquid nitrogen and stored at -80°C until RNA isolation. All procedures were carried out according to the Norwegian National Ethics Board for experimentation with animals (ID no. 4007) and current EU legislation on handling of experimental animals.

2.2 Blood haematology and biochemistry

Plasma glucose and lactate were analyzed using a Maxmat PL II autoanalyzer (ERBA Diagnostics, Montpellier, France). Plasma cortisol levels were analyzed using a EIA kit (Kit RE52061, IBL, International GmbH, Germany). The limit of detection of the assay was 2.46 ng/mL with intra- and inter-assay coefficients of variation lower than 3% and 5%, respectively. Haematocrit was measured using heparinized capillary tubes centrifuged in a Compur M1100 Microspin centrifuge (Bayer, Germany). Haemoglobin was assessed using a colorimetric kit (No 700540, Cayman Chemical Company, MI, USA).

2.3 Gene expression analysis

RNA from liver was extracted using a MagMAXTM-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA). RNA yield was 50–100 µg with 260 and 280 nm UV absorbance ratios (A260/280) of 1.9–2.1 and RIN (RNA integrity number) values of 8–10 as measured on an Agilent 2100 Bioanalyzer, which is indicative of clean and intact RNA. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Negative control

reactions were run without reverse transcriptase and real-time quantitative PCR was carried out on a CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using a 96-well PCR array layout designed for simultaneously profiling a panel of 60 genes under uniform cycling conditions (Table 1). Among the 60 genes, 40 genes were novel for gilthead sea bream and their sequences were uploaded to GenBank (JX975224–JX975265) (Supplementary file 1: Table S1). Four housekeeping genes and controls of general PCR performance were included on each array, being performed all the pipetting operations by means of the EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, RT reactions were diluted to convenient concentrations and the equivalent of 660 pg of total input RNA was used in a 25 µL volume for each PCR reaction. PCR-wells contained a 2x SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 µM were used to obtain amplicons of 50–150 bp in length (Supplementary file 2: Table S2). The program used for PCR amplification included an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. The efficiency of PCR reactions was always higher than 90%, and negative controls without sample templates were routinely performed for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5°C/10 s over a temperature range of 55–95°C), linearity of serial dilutions of RT reactions, and electrophoresis and sequencing of PCR amplified products.

Fluorescence data acquired during the PCR extension phase were normalized using the delta-delta Ct method (Livak and Schmittgen, 2001). β -actin, elongation factor 1, α -tubulin and 18S rRNA were tested for gene expression stability using GeNorm software, but the most stable gene was β -actin (M score = 0.21) and, thereby, it was used as housekeeping gene in the normalization procedure. Fold-change calculations

were done in reference to the expression ratio between ST and CTRL fish (values >1 indicate stress up-regulated genes; values <1 indicate stress down-regulated genes). For comparing the mRNA gene expression level of a panel of genes in a given stress condition, all data values were in reference to the expression level of proliferator-activated receptor gamma coactivator 1 β (PGC1 β) in CTRL fish, which was arbitrarily assigned a value of 1.

2.4 Statistical analyses

Data on fish performance, biochemistry and gene expression were analyzed using one-way analysis of variance (ANOVA-I), followed by a Student-Newman-Keuls post hoc test. When the test of normality or equal variance failed, a Mann-Whitney Rank Sum test or a Kruskal-Wallis ANOVA on ranks followed by Dunn's method was applied instead, respectively. The significance level was set at $P < 0.05$. All analyses were conducted using SPSS package version 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Fish performance

As shown in Table 2, fish exposed to thermal fluctuations (T-ST) showed an overall reduction of feed intake (nearly 40%) in comparison to CTRL fish and the other two stress groups, corresponding to changes in water levels and chasing (C-ST) and multiple sensory perceptions stressors (M-ST). This decrease in feed intake was accompanied by a statistically significant reduction ($P < 0.05$) of specific growth rates, but this detrimental effect was lower than expected due to a slight increase in feed conversion ratio (weight gain/feed intake) that was statistically significant ($P < 0.05$) for the paired stress comparisons between the T-ST and M-ST groups. C-ST fish showed similar

growth, feed utilization and feed intake as control CTRL fish. M-ST fish also showed a similar feed intake as CTRL fish, but growth rates achieved intermediate values between the two extreme groups (CTRL and T-ST) due to some detrimental effect of this type of stressor upon feed conversion.

One fish died two days before start of the stress trial, and was not replaced. On day 1 of startup one fish died in the M-ST group, and one fish died on day 20 in the C-ST group. No mortality was registered in the T-ST group.

3.2 Blood metabolic profiling

Table 2 also shows the haematological values of experimental fish. The average haematocrit value of T-ST fish was lower ($P < 0.001$) than in the other three experimental groups. A similar trend was found for the blood haemoglobin content, with the T-ST and C-ST groups becoming the two most extreme groups. No statistically significant changes were found in plasma cortisol levels, though a trend of increased cortisol titre in C-ST and M-ST fish was observed with the highest overall concentration in the latter group. No significant changes were found in plasma glucose levels regardless of the stress condition. Plasma lactate levels were statistically higher in C-ST fish than CTRL fish ($P < 0.05$). An opposite response was found in M-ST fish, and their plasma lactate values were significantly lower than CTRL and C-ST fish ($P < 0.05$). Plasma lactate levels in the T-ST group were not distinguishable from those of CTRL fish.

3.3 Mitochondrial gene expression profiling

The gene expression profile of liver mitochondria in response to intermittent and repetitive stress pulses is summarized in Supplementary file 3: Table S3. As a general

rule, repetitive thermal fluctuations triggered an up-regulated response that was statistically significant ($P < 0.05$) for one third of the genes present in the array (20 out of 60). In contrast, a slight or consistent down-regulated response affecting one or 11 genes was observed in the C-ST and M-ST groups, respectively.

For a better understanding of the results, the gene expression pattern of a given group of stressed fish was plotted against the CTRL group in a scatter plot. In the T-ST group (Fig. 1), relatively low levels of expression were found for nuclear transcription factors but, at the same time, these molecular markers were strongly up-regulated with fold-change of 5.98 for the proliferator-activated receptor gamma coactivator 1 alpha (PGC1 α), 2.32 for the nuclear respiratory factor 1 (NRF1) and 1.8 for the nuclear respiratory factor 2 (NRF2). Along with relatively high baseline levels of expression, carnitine palmitoyltransferase 1A (CPT1A) and citrate synthase (CS) were significantly up-regulated with fold changes of 4 and 1.8, respectively. Likewise, lower but statistically significant up-regulation (1.28) was observed for other closely related markers of oxidative metabolism, such as cytochrome C oxidase subunit IV isoform 1 (Cox4a). Interestingly, consistent up-regulation with fold changes ranging from 1.38 and 2.11 were also observed for most (9 out of 15) of the outer membrane translocases (TOM complex) and inner membrane translocases (TIM22 and TIM23 complexes) present in the array. Mitochondrial molecular chaperones of the Hsp10, Hsp60 and Hsp70 families were also significantly up-regulated, ranging from 1.41–1.97. More transient fold changes less than 1.45 were observed for markers of endoplasmic reticulum (ER) stress response (derlin 1, DER-1), mitochondrial dynamics (mitofusin 2, MFN2; mitochondrial fission 1 protein, FIS1), apoptosis (apoptosis-related protein 1, AIFM1) and antioxidant defense (glutathione reductase, GR).

Regarding husbandry stressors, only one gene (mitochondrial fission factor homolog B, MIFFB) of the PCR-array panel was significantly down-regulated in the C-ST group (Fig. 2). However, this down-regulated response was largely amplified in the M-ST group (Fig. 3), affecting primarily nuclear transcription factors (PGC1 α , NRF1 and NRF2) and markers of oxidative metabolism (CPT1A, CS and 3-ketoacyl-CoA thiolase, ACAA2) with fold changes ranging from 0.43 and 0.52. Similarly, a down-regulated response was detected for markers of apoptosis (apoptosis regulator BAX, BAX; Bcl-2-like protein 1, BCLX), mitochondrial dynamics (MFN2; mitochondrial Rho GTPase 2 fission, MIRO2) and inner membrane translocation (mitochondrial import inner membrane translocase subunit Tim8A).

As a corollary of the mitochondria stress profiles, the fold changes of differentially expressed genes in at least one of the three stress conditions were compiled and represented in Fig. 4. The intensity of red (up-regulated genes) and green (down-regulated genes) colors indicates the magnitude of the change.

4. Discussion

One of the great challenges of the post-genomic era is to mechanistically link genotype with phenotype (Ballard and Melvin, 2010). As the phenotype is the result of the interaction of the environment with the genotype, the definition of the triangle formed by the genome-transcriptome-environment is paramount to understand how individuals cope with external hazards and maintain homeostasis. In the present study, we analyzed the effect of environmental stressors on the phenotype of fish by integrating classical parameters of fish performance with the expression profile of mitochondrial-related genes. In this way, it is noteworthy that stressors were applied intermittently with a different periodicity, intensity and duration, and most of the genes on the mitochondrial-

array were differentially regulated, reflecting the nature of the change as well as the intensity and severity of the stressor. Thus, the cyclic thermal fluctuations, that try to mimic the natural daily changes that occur during autumn and spring in some Mediterranean regions, had the greatest detrimental effects on fish performance, and particularly in feed intake. In parallel, minor effects on growth performance and mitochondria gene expression profiling were observed with the daily lowering of water level in combination with chasing, whereas more consistent effects were observed with the set of multiple sensory perception stressors. These findings reflect the high plasticity of gilthead sea bream mitochondria when fish are faced with different stress stimuli. This becomes especially valuable for stress stimuli applied intermittently and/or at relatively low intensity levels, because it is believed that persistent and uniform rises in plasma cortisol levels are characteristic of high, but not of moderate or low stress conditions (Martínez-Porchas et al., 2009). In agreement with this, a poor consistent response of cortisol was observed in the present study, even in the group with the highest circulating cortisol concentration (M-ST group).

In mammals, mitochondrial function and activity are mainly regulated at the transcriptional level, and the mitochondrial transcription factor A (mtTFA) is a master regulator of mtDNA transcription and replication (Bengtsson et al., 2001; Gordon et al., 2001; Menshikova et al., 2006). In cultured fish the information is very scarce and we assume a high conservation of regulatory processes. In the present fish study we did not detect changes in the expression level of mtTFA mRNA after stress exposure. Nevertheless, pronounced up-regulated (T-ST group) and down-regulated (M-ST group) responses were observed in other nuclear transcription factors that modulate the expression of a number of nuclear-encoded mitochondrial proteins. Among them, attention was initially focused on NRF1 and NRF2, which tightly regulate the

mitochondrial protein import and assembly system (van Waveren and Moraes, 2008) as well as the oxidative phosphorylation (OXPHOS) pathway, including many of the ten nuclear-encoded cytochrome c oxidase subunits of complex IV of the respiratory chain (Scarpulla, 2008). A higher level of organization is represented by the family of co-activators of the peroxisome proliferator-activated receptors. The best studied member of this family is PGC1 α , which is considered a master regulator of mitochondrial biogenesis in response to several external stimuli, including caloric restriction, production of reactive oxygen species (ROS), hypoxia and thermal stress (Wenz, 2013). Accordingly, PGC1 α was observed to be the most stress-responsive gene to thermal and husbandry stressors in the present study. This agrees with the role of PGC1 α as an upstream regulator that may act in concert with NRF1 and NRF2, making them important players in fish mitochondrial biogenesis. Importantly, PGC1 β , a homologue of PGC1 α , was not specifically induced in the present study by any stressor, which may be indicative of its constitutive expression. Nevertheless, several studies in other animal models suggest potential complementary function of PGC1 α and PGC1 β , which may explain why knockouts of PGC1 α and PGC1 β are not embryonically lethal (Lin et al., 2004; Sonoda et al., 2007).

The flux of mitochondrial β -oxidation is primarily determined by carnitine palmitoyltransferase 1 (CPT1), which enables activated long chain fatty acids to enter the mitochondria (Schreurs et al., 2010). Similar to mammals, CPT1A represents the major liver isoform in fish (Britton et al., 1995; Zheng et al., 2013), and we found herein that changes in the expression level of CPT1A (T-ST and M-ST groups) mirrored variations in the mRNA transcript levels of PGC1 α and CS, a rate-determining enzyme of the tricarboxylic acid cycle commonly used as a quantitative marker of intact mitochondria (Kuzmiak et al., 2012; Trounce et al., 1996). Therefore, it appears that

both the thermal and the multiple set of sensory perception stressors induced profound changes in the oxidative capacity of mitochondria that are opposite and adaptive in nature. Indeed, the up-regulated gene expression in T-ST fish may be a counter-regulatory response to increase the oxidative capacity of fish with the drastic reduction of haematocrit and feed intake after repetitive cycles of cold exposure, which might drive a slight improvement of feed conversion as a part of a catch-up growth already reported in gilthead sea bream and other fish species (Ali et al., 2003; Ibarz et al., 2010; Montserrat et al., 2007). In contrast, low plasma lactate levels in combination with a reduced expression of mitochondrial oxidative markers would support a reduced energy demand in M-ST fish, as an adaptive response to a changing and poorly predictive environment. In the other hand, a simple rise in plasma lactate levels without any other molecular re-adjustment of mitochondrial function and activity might indicate in C-ST fish an adaptive switch from aerobic to anaerobic metabolism. Taken together all this, the expression of PGC1 α , CPT1A and CS was induced (T-ST group) or inhibited (M-ST group) in a highly coordinated manner, and it is likely that, in both fish and higher vertebrates, PGC1 α plays a key role in mitochondrial function and activity, linking mitochondrial biogenesis and energy metabolism in a highly regulated manner. Of course, further research is needed at the protein expression level to confirm and extend these findings, although it is noteworthy that microarray meta-analysis using the bioinformatics tool Fish and Chips (www.fishandchips.genouest.org/index.php) clearly show that mitochondria is among the first responders to nutritional and environmental stress stimuli in fish and gilthead sea bream in particular (Calduch-Giner et al., 2014).

A healthy metabolic phenotype is also highly dependent on the mitochondrial protein import system, which involves two assembly complexes: the translocases of the outer membrane (TOM complex) and the translocases of the inner membrane (TIM

complex). Overall this mitochondrial system has been well characterized in yeast and fungal cell systems (Bolender et al., 2008), but the components of the pathway and regulatory mechanisms remain poorly understood in mammals and practically unexplored in fish. Thus, to our knowledge, this is the first report addressing the transcriptional regulation of several components of the mitochondrial protein import system in fish. Importantly, most of the proteins subunits present in the array were highly inducible by repetitive thermal fluctuations, but not by the two husbandry stressors. In addition, it is noteworthy that major changes in mRNA transcript levels were achieved by Tom20 and Tom70 subunits, which typically share hydrophobic cytosolic domains that recognize proteins with N-terminal or internal targeting signals, respectively (Abe et al., 2000). Likewise, protein subunits of the TIM23 (Tim23, Tim44) and TIM22 (Tim9, Tim10) complexes were transcriptionally up-regulated in thermally stressed fish, mediating the targeting of proteins destined to inner membrane, inter-membrane space and the mitochondrial matrix (Sirrenberg et al., 1996). Moreover, since the TOM/TIM complex is highly inducible under conditions of chronic exercise, disease and thyroid hormone treatment, its primary action would be to ensure the maintenance of adequate protein import rates under conditions of energy deficiency (Ljubcic et al., 2010). This notion is consistent with the transcriptionally mediated response in the T-ST group, which reinforces the concept that changes in the mitochondrial protein import pathway are a normal component of the organelle response facing aerobic energy stimuli of markedly different origins.

Environmental stress might also threaten protein homeostasis by increasing the pool of unfolded and misfolded proteins. When this imbalance happens, signal transduction pathways, referred to as unfolded protein responses (UPRs), are activated in different cell compartments. The mitochondrial UPR involves the up-regulation of

mitochondrial chaperones and other factors that serve to remodel the mitochondrial-
folding environment (Broadley and Hartl, 2008). These molecular chaperones belong to
the heat shock protein families Hsp10, Hsp60 and Hsp70 (Voos, 2013). Among them, it
is generally assumed that mtHsp70 is the most important given its broad spectrum of
cellular functions, including stress response, intracellular trafficking, antigen
processing, and cell differentiation and proliferation, that make this mitochondrial
chaperone and its yeast homologue (Ssc1p) life-essential (Craig et al., 1987; Kaul et al.,
2007). In this respect, the role of mtHsp70 closely resembles the function of cytosolic
Hsp70s that is attached to the ribosomes, assisting the folding of nascent polypeptides
emerging from the ribosome exit tunnel (Peisker et al., 2010). Probably all this also
applies to fish species, as mtHsp70-deficient mutants of zebrafish have serious blood
developmental defects (Craven et al., 2005). Similarly, experimental evidence indicates
that both mtHsp70 protein and mRNA expression are highly inducible by acute and
chronic crowding stress in the liver of gilthead sea bream (Bermejo-Nogales et al.,
2008; Pérez-Sánchez et al., 2013). This assumption was further reinforced herein, where
repetitive thermal fluctuations were able to induce the expression of mtHsp70 following
changes in the expression level of Tim44, a component of the inner membrane TIM23
translocase complex that works in yeast in the immediate vicinity of mtHsp70 (Rassow
et al., 1994).

Other mitochondrial chaperones, annotated as 40 kDa heat shock protein DnaJ
homolog (DnaJA3A) and iron-sulphur cluster co-chaperone protein HscB (DnaJC20),
were not significantly altered by any of the stressors considered in the present study.
However, a consistent and coordinated up-regulation in response to thermal fluctuations
was observed for Hsp60 and co-chaperone Hsp10, which might act in a sequential order
with the mtHsp70 as pointed out by Voos and Röttgers (2002) in yeast. According to

this, pre-folded mitochondrial proteins first encounter mtHsp70 and, only after being subsequently released from mtHsp70, these pre-proteins interact with the Hsp60/Hsp10 complex to become functionally active. At the present time, it is difficult to evaluate the magnitude of the response induced by repetitive thermal fluctuations on the mitochondrial translocase/chaperone system of gilthead sea bream, but it is noteworthy that the ER stress response was limited to a relatively low response of DER-1, whereas no response was found for other highly stress-responsive markers (Hsp40 co-chaperone, 170 kDa glucose-regulated protein) of cell-tissue repair in the ER of gilthead sea bream (Calduch-Giner et al., 2010; Pérez-Sánchez et al., 2013). This observation strongly supports that mitochondria rather than ER are especially sensitive to intermittent and repetitive stress disturbances, such as common natural stressors that mimic natural changes in water temperature.

The transcriptionally mediated changes in the chaperone and protein import system were mainly induced in the T-ST group, whereas molecular markers of mitochondrial dynamics and apoptosis were altered either by thermal or husbandry stressors, represented by the M-ST group. The machinery involved in mitochondria shaping results from the balance of two opposing processes (fusion and fission), but it may also be greatly affected by the “railways” used by mitochondria to move inside the cell, which suggests a cross-talk between cytoskeletal and mitochondrial fusion/fission proteins. Importantly, this process seems to be highly conserved in yeast and mammals (Anesti and Scorrano, 2006), and we report herein that the nucleotide sequence of four major components of the fusion (MFN1, MFN2) and fission (FIS1, MIFFB) system possess a high degree of identity (E-value < 5e-68) to the homologous proteins in mammals. The same is applicable (E-value = 0) to proteins of the MIRO system (MIRO1A, MIRO2) involved in mitochondrial movements through the cell

(Supplementary file 1: Table S1). As a general rule, these processes enable mitochondria to mix their contents within the cell network, allowing the redistribution of mitochondria, simultaneously increasing oxidative capacity, which is advantageous under conditions of high energy demand. Conversely, mitochondria fission or fragmentation is a mechanism that segregates components of the mitochondria network that are dysfunctional or damaged, allowing their removal. Hence, the dynamic regulation of fusion and fission events adapts mitochondria morphology to the bioenergetic requirements of the cell (Liesa et al., 2009; Romanello and Sandri, 2013). In this regard, several observations in the present study support the notion that MFN2 is induced by PGC1 α and estrogen receptor- α in response to exercise, cold exposure and β -adrenergic agonists (Slivka et al., 2012; Soriano et al., 2006). In agreement with this, we found that repeated exposure to cyclic drops in water temperature enhanced the expression of PGC1 α and MFN2, whereas the down-regulated expression of PGC1 α was concurrent with the transcriptional down-regulation of MFN2 in the M-ST group. In parallel, the overexpression of mitochondrial fission protein FIS1 in the T-ST group indicates attempts to promote autophagy of damaged mitochondria in the cell via increased fission pathways. In fact, overexpression of FIS1 induces apoptosis in different cell culture systems, which suggests that mitochondrial fission may be a driver of apoptosis (Alirol et al., 2006; Baltzer et al., 2010; James et al., 2003; Wallace and Fan, 2010; Yu et al., 2005). However, FIS1 mediated cell death is inhibited by the anti-apoptotic Bcl-xL overexpression, demonstrating that the cells die due to extensive mitochondrial dysfunction rather than fission-induced mitochondrial permeabilisation (Alirol et al., 2006; Yu et al., 2005). In any case, as reported for fusion/fission processes, the dualism of apoptotic and anti-apoptotic processes seems to be a characteristic feature of the complex mitochondrial trade-off and, in the present study,

the expression of both anti-apoptotic (e.g. BCLX) and apoptotic factors (e.g. BAX) was significantly repressed at the same level in the M-ST group.

In conclusion, our results highlight for the first time in fish the transcriptional plasticity of most nuclear-encoded mitochondria proteins that affect a vast array of processes, including mitochondrial biogenesis and oxidative metabolism, mitochondrial protein import/folding/assembly, as well as mitochondrial dynamics and apoptosis. Importantly, most of the genes on the array were differentially regulated by repetitive exposure to natural and husbandry stressors, and the magnitude of the mitochondrial transcriptionally-mediated changes reflects the intensity and severity of the stressor. Thus, the present study revealed new insights on the capacity of fish to efficiently manage “allostatic load”, defined as the process that maintains stability through change of a number of stress mediators. The ultimate physiological consequences are still under investigation but, as summarized in Fig. 5, the gene expression profile of fish exposed to the repetitive cycling of water temperature indicates that a reactive mitochondrial phenotype helps to increase the aerobic oxidative capacity of fish. In contrast, in the C-ST group, an apparent lack of mitochondria response in combination with increased lactate production is indicative of some kind of metabolic switch that primes the anaerobic metabolism in response to short periods of increased energy demand that do not have a major impact on fish performance. The third response pattern, with the highest theoretical allostatic load score, is represented by the M-ST group, in which the overall down-regulation of mitochondrial-related genes in combination with decreased lactate production is indicative of reduced energy demand and oxidative metabolic capacity, leading to the impairment of feed conversion in a changing and poor predictive milieu.

Abbreviations

ACAA2: 3-ketoacyl-CoA thiolase, mitochondrial; AIFM1: Apoptosis-related protein 1; AIFM3, Apoptosis-related protein 3; ANOVA: analysis of variance; BAX: Apoptosis regulator BAX; BCL2: Apoptosis regulator Bcl-2; BCLX: Bcl-2-like protein 1; CAT: Catalase; Cox4a: Cytochrome C oxidase subunit IV isoform 1; CPT1A: Carnitine palmitoyltransferase 1A; CS: Citrate synthase; DER-1: Derlin-1; DnaJA3Aa: 40 KDa heat shock protein DnaJ (Hsp40) homolog, subfamily A, member 3A; DnaJC20: Iron-sulfur cluster co-chaperone protein HscB; ECH: Enoyl-CoA hydratase, mitochondrial; ER: endoplasmic reticulum; ERdj3: ER-associated Hsp40 co-chaperone; FIS1: Mitochondrial fission 1 protein; GPX4: Glutathione peroxidase 4; GR: Glutathione reductase; Grp-170: 170 kDa Glucose-regulated protein; GST3: Glutathione S-transferase 3; HADH: Hydroxyacyl-CoA dehydrogenase; IDH3A: Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial; IDH3B: Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial; IDH3G: Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial; MFN1: Mitofusin 1; MFN2: Mitofusin 2; MIFFB: Mitochondrial fission factor homolog B; MIRO1A: Mitochondrial Rho GTPase 1; MIRO2: Mitochondrial Rho GTPase 2; mtHsp10: 10 kDa heat shock protein, mitochondrial; mtHsp60: 60 KDa heat shock protein, mitochondrial; mtHsp70: 70 kDa heat shock preotin, mitochondrial; mtTFA: Transcription factor A, mitochondrial; NRF1: Nuclear respiratory factor 1; NRF2: Nuclear respiratory factor 2; OXPHOS: oxidative phosphorylation; PERP: p53 apoptosis effector related to PMP-22; PGC1 α : Proliferator-activated receptor gamma coactivator 1 alpha; PGC1 β : Proliferator-activated receptor gamma coactivator 1 beta; PRDX3: Peroxiredoxin 3; PRDX5: Peroxiredoxin 5; ROS: reactive oxygen species; SOD2: Superoxide dismutase [Mn]; Tim10: Translocase of inner mitochondrial membrane 10 homolog; Tim13:

Mitochondrial import inner membrane translocase subunit 13; Tim14: Mitochondrial import inner membrane translocase subunit 14; Tim16: Mitochondrial import inner membrane translocase subunit 16; Tim17A: Mitochondrial import inner membrane translocase subunit Tim17-A; Tim22: Mitochondrial import inner membrane translocase subunit Tim22; Tim23: Mitochondrial import inner membrane translocase subunit 23; Tim44: Mitochondrial import inner membrane translocase subunit Tim44; Tim8A: Mitochondrial import inner membrane translocase subunit Tim8 A; Tim9: Mitochondrial import inner membrane translocase subunit Tim9; Tom22: Mitochondrial import receptor subunit Tom22; Tom34: Mitochondrial import receptor subunit Tom34; Tom5: Mitochondrial import receptor subunit Tom5 homolog; Tom7: Mitochondrial import receptor subunit Tom7 homolog; Tom70: Mitochondrial import receptor subunit Tom70; UCP1: Uncoupling protein 1; UCP2: Uncoupling protein 2; UCP3: Uncoupling protein 3; UPR: unfolded protein response.

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Supplementary data

Supplementary file 1: Table S1. Characteristics of new assembled sequences

according to BLAST searches.

Supplementary file 2: Table S2. Forward and reverse primers for real-time PCR.

Supplementary file 3: Table S3. Effect of three types of stressors on the expression of

liver mitochondrial-related genes. CTRL, control group; T-ST, thermal stress group; C-

ST, chasing stress group; M-ST, multiple sensory perception stress group. Values are

the mean $\pm$ SEM (n = 6-8). Rows with unlike superscript letters were significantly

different (P<0.05; Student-Newman-Keuls).

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788

Table 1. PCR-array layout of 60 genes with extra-wells for housekeeping genes and general controls of PCR performance.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Hsp10	CAT	CPT1A	COX4a	Tom5	Tim22	MIRO2	NRF1	ACTB	ACTB	PPC1	PPC1
B	DnaJA3A	GPX4	CPT1B	UCP1	Tim44	Tim10	AIFM1	NRF2	EF-1	EF-1	PPC2	PPC2
C	DnaJC20	GR	ECH	UCP2	Tim23	Tim9	AIFM3	PGC1 α	α -tubulin	α -tubulin	PPC3	PPC3
D	mtHsp60	GST3	HADH	UCP3	Tim17A	FIS1	BAX	PGC1 β	18S rRNA	18S rRNA	PPC4	PPC4
E	mtHsp70	PRDX3	CS	Tom70	Tim16	MIFFB	BCL2				NPC	NPC
F	DER-1	PRDX5	IDH3A	Tom34	Tim14	MFN1	BCLX					
G	ERdj3	SOD2	IDH3B	Tom22	Tim13	MFN2	PERP					
H	Grp170	ACAA2	IDH3G	Tom7	Tim8A	MIRO1A	mtTFA					

Position	Symbol	Description	Accession No.
A1	mtHsp10	10 kDa heat shock protein, mitochondrial	JX975224
B1	DnaJA3A	40 kDa heat shock protein DnaJ (Hsp40) homolog, member 3A	JX975225
C1	DnaJC20	Iron-sulfur cluster co-chaperone protein HscB, mitochondrial	JX975226
D1	mtHsp60	60 kDa heat shock protein, mitochondrial	JX975227
E1	mtHsp70	70 kDa heat shock protein, mitochondrial	DQ524993
F1	DER-1	Derlin-1	JQ308825
G1	ERdj3	ER-associated Hsp40 co-chaperone	JQ308827
H1	Grp170	170 kDa glucose-regulated protein	JQ308821
A2	CAT	Catalase	JQ308823
B2	GPX4	Glutathione peroxidase 4	AM977818
C2	GR	Glutathione reductase	AJ937873
D2	GST3	Glutathione S-transferase 3	JQ308828
E2	PRDX3	Peroxiredoxin 3	GQ252681
F2	PRDX5	Peroxiredoxin 5	GQ252683
G2	SOD2	Superoxide dismutase [Mn]	JQ308833
H2	ACAA2	3-ketoacyl-CoA thiolase, mitochondrial	JX975228
A3	CPT1A	Carnitine palmitoyltransferase 1A	JQ308822
B3	CPT1B	Carnitine palmitoyltransferase 1B	DQ866821
C3	ECH	Enoyl-CoA hydratase, mitochondrial	JQ308826
D3	HADH	Hydroxyacyl-CoA dehydrogenase	JQ308829
E3	CS	Citrate synthase	JX975229
F3	IDH3A	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	JX975231
G3	IDH3B	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	JX975232
H3	IDH3G	Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial	JX975233
A4	Cox4a	Cytochrome C oxidase subunit IV isoform 1	JQ308835
B4	UCP1	Uncoupling protein 1	FJ710211
C4	UCP2	Uncoupling protein 2	JQ859959
D4	UCP3	Uncoupling protein 3	EU555336
E4	Tom70	Mitochondrial import receptor subunit Tom70	JX975234
F4	Tom34	Mitochondrial import receptor subunit Tom34	JX975235
G4	Tom22	Mitochondrial import receptor subunit Tom22	JX975236
H4	Tom7	Mitochondrial import receptor subunit Tom7 homolog	JX975237
A5	Tom5	Mitochondrial import receptor subunit Tom5 homolog	JX975238
B5	Tim44	Mitochondrial import inner membrane translocase subunit 44	JX975239
C5	Tim23	Mitochondrial import inner membrane translocase subunit 23	JX975240
D5	Tim17A	Mitochondrial import inner membrane translocase subunit 17A	JX975241

Table 1. Continued.

Position	Symbol	Description	Accession No.
E5	Tim16	Mitochondrial import inner membrane translocase subunit 16	JX975242
F5	Tim14	Mitochondrial import inner membrane translocase subunit Tim14	JX975243
G5	Tim13	Mitochondrial import inner membrane translocase subunit Tim13	JX975244
H5	Tim8A	Mitochondrial import inner membrane translocase subunit Tim8A	JX975245
A6	Tim22	Mitochondrial import inner membrane translocase subunit Tim22	JX975246
B6	Tim10	Mitochondrial import inner membrane translocase subunit Tim10	JX975247
C6	Tim9	Mitochondrial import inner membrane translocase subunit Tim9	JX975248
D6	FIS1	Mitochondrial fission 1 protein	JX975249
E6	MIFFB	Mitochondrial fission factor homolog B	JX975252
F6	MFN1	Mitofusin 1	JX975250
G6	MFN2	Mitofusin 2	JX975251
H6	MIRO1A	Mitochondrial Rho GTPase 1	JX975253
A7	MIRO2	Mitochondrial Rho GTPase 2	JX975254
B7	AIFM1	Apoptosis-related protein 1	JX975255
C7	AIFM3	Apoptosis-related protein 3	JX975256
D7	BAX	Apoptosis regulator BAX	JX975257
E7	BCL2	Apoptosis regulator Bcl-2	JX975258
F7	BCLX	Bcl-2-like protein 1	JX975259
G7	PERP	p53 apoptosis effector related to PMP-22	JX975260
H7	mtTFA	Mitochondrial transcription factor A	JX975262
A8	NRF1	Nuclear respiratory factor 1	JX975263
B8	NRF2	Nuclear respiratory factor 2	JX975261
C8	PGC1 α	Proliferator-activated receptor gamma coactivator 1 alpha	JX975264
D8	PGC1 β	Proliferator-activated receptor gamma coactivator 1 beta	JX975265
A9, A10	ACTB	β -actin	X89920
B9, B10	EF-1	Elongation factor 1	AF184170
C9, C10	α -tubulin	α -tubulin	AY326430
D9, D10	18S rRNA	18S ribosomal RNA	AY993930
A11-D11	PPC1/PPC4	Positive PCR control (serial dilutions of standard gene)	AY590304
A12-D12	PPC1/PPC4	Positive PCR control (serial dilutions of standard gene)	AY590304
E11, E12	NPC	Negative PCR control	

Mitochondrial chaperones: mtHsp10, DnaJA3A, DnaJC20, mtHsp60, mtHsp70

Endoplasmic reticulum stress response: DER-1, ERdj, GRP-170

Antioxidant defense: CAT, GPX4, GR, GST3, PRDX3, PRDX5, SOD2

Oxidative metabolism: ACAA2, CPT1A, CPT1B, ECH, HADH, CS, IDH3A, IDH3B, IDH3G, Cox4a

Mitochondrial respiration uncoupling: UCP1, UCP2, UCP3

Outer membrane translocases (TOM complex): Tom70, Tom34, Tom22, Tom7, Tom5

Inner membrane translocases (TIM23 complex): Tim44, Tim23, Tim17A, Tim16, Tim14, Tim13, Tim8A

Inner membrane translocases (TIM22 complex): Tim22, Tim10, Tim9

Mitochondrial dynamics: FIS1, MIFFB, MFN1, MFN2, MIRO1A, MIRO2

Apoptosis: AIFM1, AIFM3, BAX, BCL2, BCLX

Nuclear transcription factors: mtTFA, NRF1, NRF2, PGC1 α , PGC1 β

Housekeeping genes: ACTB, EF-1, α -tubulin, 18S rRNA

Table 2. Data on growth performance and plasma biochemistry and haematology of fish exposed to stress stimuli. Thermal stress (T-ST), chasing stress (C-ST) and multiple sensory perception stress (M-ST). Data on growth performance are the mean $\pm$ SEM of triplicate tanks. Cortisol levels are the mean of 9 fish (3 fish per triplicated tank). Other systemic measurements are the mean of 20-24 animals (8-6 fish per triplicated tank).

	CTRL	T-ST	C-ST	M-ST	P ¹
Initial body weight (g)	261.0 $\pm$ 1.6	252.1 $\pm$ 4.6	255.38 $\pm$ 3.9	259.4 $\pm$ 2.2	0.30
Final body weight (g)	329.1 $\pm$ 0.70 ^a	297.9 $\pm$ 7.1 ^b	319.79 $\pm$ 8.6 ^{ab}	316.47 $\pm$ 4.4 ^{ab}	0.03
Feed intake (%)	0.56 $\pm$ 0.02 ^a	0.37 $\pm$ 0.01 ^b	0.55 $\pm$ 0.01 ^a	0.56 $\pm$ 0.02 ^a	<0.001
FCR ²	1.02 $\pm$ 0.04 ^{ab}	1.18 $\pm$ 0.02 ^a	1.00 $\pm$ 0.04 ^{ab}	0.87 $\pm$ 0.07 ^b	0.03
SGR (%) ³	0.58 $\pm$ 0.01 ^a	0.42 $\pm$ 0.03 ^b	0.56 $\pm$ 0.04 ^{ab}	0.49 $\pm$ 0.06 ^{ab}	0.05
Haematocrit (%)	35.3 $\pm$ 0.8 ^a	28.5 $\pm$ 1.2 ^b	37.5 $\pm$ 1.16 ^a	34.5 $\pm$ 0.81 ^a	<0.001
Haemoglobin (g/dL)	11.0 $\pm$ 0.27 ^a	9.5 $\pm$ 0.43 ^b	12.3 $\pm$ 1.2 ^a	10.6 $\pm$ 0.24 ^{ab}	0.005
Plasma cortisol (ng/mL)	10.4 $\pm$ 2.2	10.7 $\pm$ 1.9	17.2 $\pm$ 2.9	37.2 $\pm$ 19.7	0.24
Plasma glucose (mM/L)	3.8 $\pm$ 0.29	3.8 $\pm$ 0.25	3.9 $\pm$ 0.26	3.7 $\pm$ 0.3	0.93
Plasma lactate (mM/L)	2.78 $\pm$ 0.18 ^b	2.91 $\pm$ 0.34 ^{ab}	3.3 $\pm$ 0.18 ^a	2.0 $\pm$ 0.18 ^c	0.05

¹P values result from analysis of variance. Different superscript letters in each row indicate significant differences among experimental groups (Student Newman-Keuls test, P < 0.05)

²Feed conversion ratio = weight gain / feed intake

³Specific growth rate = [100 $\times$ (ln final fish weight – ln initial fish weight)] / days

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Figure captions

Fig. 1. Mitochondria gene expression profile of fish exposed to thermal fluctuations (T-ST group). Relative mRNA expression levels are plotted against the expression values from control fish (CTRL). Data are the mean of 6–8 fish (for details of standard errors see Supplementary file 3: Table S3). β -actin was used as a housekeeping gene, and all data values in the scatterplot are relative to the expression level of PGC1 β in CTRL fish. For differentially expressed genes, fold change calculations for a given gene were done using data from CTRL as arbitrary reference values (values >1 indicate stress up-regulated genes).

Fig. 2. Mitochondria gene expression profile of fish exposed to changes in water levels and chasing (C-ST group). Relative mRNA expression levels are plotted against the expression values from control fish (CTRL). Data are the mean values of 6–8 fish (for details of standard errors see Supplementary file 3: Table S3). β -actin was used as a housekeeping gene, and all data values in the scatterplot are relative to the expression level of PGC1 β in CTRL fish. For differentially expressed genes, fold change calculations for a given gene were done using data from CTRL as arbitrary reference values (values <1 indicate stress down-regulated genes).

Fig. 3. Mitochondria gene expression of fish exposed to multiple sensory perception stressors (M-ST group). Relative mRNA expression levels are plotted against the expression values from control fish (CTRL). Data are the mean values of 6–8 fish (for details of standard errors see Supplementary file 3: Table S3). β -actin was used as a housekeeping gene, and all data values in the scatterplot are relative to the expression level of PGC1 β in CTRL fish. For differentially expressed genes, fold change

calculations for a given gene were done using data from CTRL as arbitrary reference values (values <1 indicate stress down-regulated genes).

Fig. 4. Corollary of mitochondria gene expression profiles of differentially expressed genes. Fish were exposed to intermittent and repetitive natural and husbandry stress stimuli: thermal stress (T-S1), chasing stress (C-ST) and multiple sensory perceptions stress (M-ST). Fold changes are relative to the control group (CTRL). Red tones correspond to up-regulated genes and green tones correspond to down-regulation. The intensity of the colour represents the degree of change. Statistically significant differences between CTRL and stressed groups are indicated (*, $P < 0.05$; Student *t*-test).

Fig. 5. Integrative physiological response of fish. This response includes growth performance, plasma biochemistry, haematology, mitochondrial activity and biogenesis with a given allostatic load score of fish exposed to natural and husbandry stress stimuli, including thermal stress (T-ST), chasing stress (C-ST) and multiple sensory perceptions stress (M-ST).

Genes	Fold change (T-ST/CTRL)
PGC1α	5.98
NRF1	2.32
NRF2	1.80
CPT1A	4.00
CS	1.81
COX4a	1.28
Tom22	2.11
Tim10	1.72
Tom70	1.61
Tim9	1.61
Tim44	1.45
Tom34	1.44
Tim23	1.38
mtHsp60	1.97
mtHsp10	1.85
mtHsp70	1.41
DER-1	1.35
MFN2	1.42
FIS1	1.32
AIFM1	1.24
GR	1.22

Figure 1

Genes	Fold change (C-ST/CTRL)
MIFFB	0.70

Figure 2

Genes	Fold change (M-ST/CTRL)
PGC1α	0.43
NRF1	0.71
NRF2	0.72
CPT1A	0.52
CS	0.64
ACAA2	0.66
BAX	0.62
BCLX	0.68
MFN2	0.69
MIRO2	0.83
Tim8A	0.73

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		STRESS GROUP	T-ST	C-ST	M-ST
Nuclear transcription factors	{	PGC1 α	5.98*	1.24	0.43*
		NRF1	2.32*	0.88	0.71*
		NRF2	1.8	0.86	0.72
Oxidative metabolism markers	{	CPT1A	4*	0.91	0.52*
		ACAA2	0.97	0.8	0.66*
		CS	1.81*	0.9	0.64*
Outer membrane translocation	{	COX4a	1.28*	0.95	1.03
		Tom70	1.61*	0.95	0.97
		Tom34	1.44*	1.03	0.84
Inner membrane translocases (TIM23 complex)	{	Tom22	2.11*	1.29	1.43
		Tim44	1.45*	1.13	0.89
		Tim23	1.38*	1.27	1
Inner membrane translocases (TIM22 complex)	{	Tim8A	1.04	0.86	0.73*
		Tim10	1.72*	0.93	0.96
		Tim9	1.61*	0.96	0.83
Molecular chaperones	{	mtHsp10	1.85*	1.19	0.8
		mtHsp60	1.97*	0.86	0.79
		mtHsp70	1.41*	0.98	0.87
Antioxidant enzyme	{	DER-1	1.35*	1.13	0.88
		GR	1.22*	0.97	1.01
		FIS1	1.32*	1	0.91
Fusion & Fission markers	{	MFN2	1.42*	0.87	0.69*
		MIFFB	0.92	0.7*	0.73
		MIRO2	1.03	1.08	0.83*
Apoptotic markers	{	AIFM1	1.24*	0.87	1.15
		BAX	1.14	0.84	0.62*
		BCLX	1.1	0.83	0.68*

Figure 4

Feed intake

Growth

Feed conversion

Hc-Hb

Cortisol

Lactate

Mitochondrial activity & Biogenesis

Adaptive stress response

Allostatic load score

891 **Table S1.** Characteristics of new assembled sequences according to BLAST searches.

Contigs	F ^a	Size (nt)	Annotation ^b	Best match ^c	E ^d	CDS ^e
C2_4023	95	570	mtHsp10	ACQ58985	2e-55	98-397
C2_6932	96	2165	DnaJA3A	XP_003450123	0	33-1397
C2_4907	110	1192	DnaJC20	XP_003454205	9e-111	243-968
C2_5222	145	3195	mtHsp60	ADM73510	0	123-1862
C2_1174	472	1864	ACAA2	XP_003451083	0	131-1321
C2_2740	171	3386	CS	Q6S9V7	0	124-1533
C2_5093	53	1542	IDH3A	XP_003440485	0	55-1212
C2_1275	295	1650	IDH3B	CBN81104	0	53-1201
C2_3444	204	2138	IDH3G	XP_003448211	0	43-1233
C2_22819	13	976	Tom70	XP_003452621	0	<1->976
C2_17904	30	1398	Tom34	ACI33761	8e-123	140-1072
C2_3036	199	1588	Tom22	ACO09752	6e-36	209-610
C2_4825	51	565	Tom7	CAF89564	1e-30	70-237
C2_5265	74	736	Tom5	ACQ58779	5e-13	113-268
C2_15041	40	777	Tim44	XP_003439971	5e-120	<1->777
C2_4029	124	1606	Tim23	CBN81624	5e-104	308-943
C2_138	777	1364	Tim17A	CBN80814	1e-82	45-551
C2_15083	34	556	Tim16	NP_957098	2e-40	105-476
C2_5885	44	611	Tim14	ACO07830	3e-41	121-471
C2_2920	189	1609	Tim13	ACQ58319	7e-46	112-399
C2_1464	245	891	Tim8A	XP_003457647	8e-49	190-459
C2_12934	24	1018	Tim22	XP003456212	5e-99	20-625
C2_8198	87	515	Tim10	XP_003448035	1e-48	140-406
C2_7203	64	890	Tim9	NP_001153383	3e-51	75-344
C2_198	1062	1200	FIS1	XP_003449392	5e-68	33-497
C2_1143	198	1369	MIFFB	XP_003441668	9e-106	217-909
C2_7180	62	2455	MFN1	CAG08068	0	<1-1355
C2_11777	44	2215	MFN2	XP_003459870	0	482->2215
C2_9297	66	2398	MIRO1A	XP_003452865	0	362-2221
C2_3084	160	3257	MIRO2	CBN81307	0	162-2018
C2_6260	91	1715	AIFM1	XP_003456194	0	<1-1328
C2_358	651	1386	AIFM3	ACQ58260	7e-139	41-736
C2_2702	178	2082	BAX	CAG02784	1e-109	206-784
C2_23085	12	1056	BCL-2	XP_003437950	6e-100	548->1056
C2_7453	45	1321	BCL2L	CBN81010	3e-96	243-890
C2_2885	200	1139	PERP	NP_001135192	3e-70	227-784
C2_1902	207	2381	mtTFA	ACQ58415	1e-104	180-1058
C2_23517	16	1297	NRF1	XP_003445010	1e-31	<1-267
C2_11237	36	1092	NRF2	XP_003452933	0	145->1092
C2_43962	5	252	PGC1 α	CAG02304	1e-20	25->252
C2_65322	7	770	PGC1 β	XP_003447675	2e-88	<1-500

892 ^a Number of reads composing the assembled sequences.

893 ^b Gene identity determined through BLAST searches: mtHsp10, 10 kDa heat shock protein, mitochondrial;
894 DnaJA3A, 40 kDa heat shock protein DnaJ (Hsp40) homolog, subfamily A, member 3A; DnaJC20, Iron-
895 sulfur cluster co-chaperone protein HscB; mtHsp60, 60 kDa heat shock protein, mitochondrial; ACAA2, 3-
896 ketoacyl-CoA thiolase, mitochondrial; CS, Citrate synthase; IDH3A, Isocitrate dehydrogenase [NAD]
897 subunit alpha, mitochondrial; IDH3B, Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial;
898 IDH3G, Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial; Tom70, Mitochondrial import
899 receptor subunit Tom70; Tom34, Mitochondrial import receptor subunit Tom34; Tom22, Mitochondrial
900 import receptor subunit Tom22; Tom7, Mitochondrial import receptor subunit Tom7 homolog; Tom5,
901 Mitochondrial import receptor subunit Tom5 homolog; Tim44, Mitochondrial import inner membrane
902 translocase subunit Tim44; Tim23, Mitochondrial import inner membrane translocase subunit 23; Tim17A,

903 Mitochondrial import inner membrane translocase subunit Tim17-A; Tim16, Mitochondrial import inner
 904 membrane translocase subunit 16; Tim14, Mitochondrial import inner membrane translocase subunit 14;
 905 Tim13, Mitochondrial import inner membrane translocase subunit 13; Tim8A, Mitochondrial import inner
 906 membrane translocase subunit Tim8A; Tim22, Mitochondrial import inner membrane translocase subunit
 907 Tim22; Tim10, Translocase of inner mitochondrial membrane 10 homolog; Tim9, Mitochondrial import
 908 inner membrane translocase subunit Tim9; FIS1, Mitochondrial fission 1 protein; MIFFB, Mitochondrial
 909 fission factor homolog B; MFN1, Mitofusin 1; MFN2, Mitofusin 2; MIRO1A, Mitochondrial Rho GTPase
 910 1; MIRO2, AIFM1, Apoptosis-related protein 1; AIFM3, Apoptosis-related protein 3; BAX, Apoptosis
 911 regulator BAX; Bcl-2, Apoptosis regulator Bcl-2; BCL2L, Bcl-2-like protein 1; PERP, p53 apoptosis
 912 effector related to PMP-22; Mitochondrial Rho GTPase 2; mtTFA, Transcription factor A, mitochondrial;
 913 NRF1, Nuclear respiratory factor 1; NRF2, Nuclear respiratory factor 2; PGC1 α , Proliferator-activated
 914 receptor gamma coactivator 1 alpha; PGC1 β , Proliferator-activated receptor gamma coactivator 1 beta.
 915 ^c Best BLAST-X protein sequence match (lowest E value).

916 ^d Expectation value.

917 ^e Codifying domain sequence.

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Table S2. Forward and reverse primers for real-time PCR.

Gene name	Symbol	Primer sequence
β-actin	ACTB	F TCC TGC GGA ATC CAT GAG A R GAC GTC GCA CTT CAT GAT GCT
Elongation factor 1	EF-1	F CCC GCC TCT GTT GCC TTC G R CAG CAG TGT GGT TCC GTT AGC
α-tubulin	α-tubulin	F GAC ATC ACC AAT GCC TGC TTC R GTG GCG ATG GCG GAG TTC
18S ribosomal RNA	18S r RNA	F GCA TTT ATC AGA CCC AAA ACC R AGT TGA TAG GGC AGA CAT TCG
10 kDa heat shock protein, mitochondrial	mtHsp10	F CAT GCT GCC AGA GAA GTC TCA AGG R AGG TCC CAC TGC CAC TAC TGT
40 kDa heat shock protein DnaJ (Hsp40) homolog, subfamily A, member 3A	DnaJA3A	F CCA AAT GCT GTC TCC TCA CTG TCC TTT C R ACC TGA TAG AAG TCC TGC TTG CTG CTA
Iron-sulfur cluster co-chaperone protein HscB	DnaJC20	F GCC AGA AGC AGC CAA TAG GAT R CTT TGA GCA GGG CAG CGT CTA
60 kDa heat shock protein, mitochondrial	Hsp60	F TGT GGC TGA GGA TGT GGA TGG AGA G R GCC TGT TGA GAA CCA AGG TGC TGA G
70 kDa heat shock protein, mitochondrial	mtHsp70	F TCC GGT GTG GAT CTG ACC AAA GAC R TGT TTA GGC CCA GAA GCA TCC ATG
Derlin-1	DER-1	F ACT GCC TCG GTT GCC TTT CC R TGG CTG TCA CAA GTC TCC AGA TAT G
ER-associated Hsp40 co-chaperone	ERdj3	F AAC CGA CAG CAG CAG GAC AG R ACT TCT TCA AGC GTG ACC TCC AG
170 kDa glucose-regulated protein	Grp-170	F CAG AGG AGG CAG ACA GCA AGA C R TTC TCA GAC TCA GCA TTT CCA GAT TTC
Catalase	CAT	F TGG TCG AGA ACT TGA AGG CTG TC R AGG ACG CAG AAA TGG CAG AGG
Glutathione peroxidase 4	GPX4	F TGC GTC TGA TAG GGT CCA CTG TC R GTC TGC CAG TCC TCT GTC GG
Glutathione reductase	GR	F TGT TCA GCC ACC CAC CCA TCG G R GCG TGA TAC ATC GGA GTG AAT GAA GTC TTG
Glutathione S-transferase 3	GST3	F CCA GAT GAT CAG TAC GTG AAG ACC GTC R CTG CTG ATG TGA GGA ATG TAC CGT AAC
Peroxiredoxin 3	PRDX3	F ATC AAC ACC CCA CGC AAG ACT G R ACC GTT TGG ATC AAT GAG GAA CAG ACC
Peroxiredoxin 5	PRDX5	F GAG CAC GGA ACA GAT GGC AAG G R TCC ACA TTG ATC TTC TTC ACG ACT CC
Superoxide dismutase [Mn]	SOD2	F CCT GAC CTG ACC TAC GAC TAT GG R AGT GCC TCC TGA TAT TTC TCC TCT G
3-ketoacyl-CoA thiolase, mitochondrial	ACAA2	F CAT CAC TGC CCA CCT GGT TCA T R CCA ACA GCG TAC TTG CCT CCT
Carnitine palmitoyltransferase 1A	CPT1A	F GTG CCT TCG TTC GTT CCA TGA TC R TGA TGC TTA TCT GCT GCC TGT TTG
Carnitine palmitoyltransferase 1B	CPT1B	F CCA CCA GCC AGA CTC CAC AG R CAC CAC CAG CAC CCA CAT ATT TAG
Enoyl-CoA hydratase, mitochondrial	ECH	F GCC CAA GAA GCC AAG CAA TCA G R CTT TAG CCA TAG CAG AGA CCA GTT TG

Gene name	Symbol	Primer sequence
<u>Hydroxyacyl-CoA dehydrogenase</u>	HADH	F GAA CCT CAG CAA CAA GCC AAG AG R CTA AGA GGC GGT TGA CAA TGA ATC C
<u>Citrate synthase</u>	CS	F TCC AGG AGG TGA CGA GCC R GTG ACC AGC AGC CAG AAG AG
<u>Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial</u>	IDH3A	F CCA CCC ATC TAT GAA CCT GCT GCT GAG R CAC ACA CGG ACG CAC ATT GGC ATA
<u>Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial</u>	IDH3B	F CCT CGG TCT GTT CAC GGA TGA TGA R CAG CAC TCG CCA CAA CAA CCT
<u>Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial</u>	IDH3G	F GCT TAG ACC TCT ATG CGA ATG TGA TG R TGT CAA TGT TCT TGT GGC GAG TC
<u>Cytochrome C oxidase subunit IV isoform 1</u>	Cox4a	F ACC CTG AGT CCA GAG CAG AAG TCC R AGC CAG TGA AGC CGA TGA GAA AGA AC
<u>Uncoupling protein 1</u>	UCP1	F GCA CAC TAC CCA ACA TCA CAA G R CGC CGA ACG CAG AAA CAA AG
<u>Uncoupling protein 2</u>	UCP2	F CGG CGG CGT CCT CAG TTG R AAG CAA GTG GTC CCT CTT TGG TCA T
<u>Uncoupling protein 3</u>	UCP3	F AGG TGC GAC TGG CTG ACG R TTC GGC ATA CAA CCT CTC CAA AG
<u>Mitochondrial import receptor subunit Tom70</u>	Tom70	F GAG TCA GGT GGT CGA TAC A R CCA ATG AGC AGG TAG AAT GTG
<u>Mitochondrial import receptor subunit Tom34</u>	Tom34	F GCT ACC GCC ACT TCT CCA CAA R TCT GTT TGG TGC CGT TCT GCT
<u>Mitochondrial import receptor subunit Tom22</u>	Tom22	F CGC TCT GGG TGG GTA CTA CCT CCT T R CGA ACA CAA CAG GCA GCA CCA GGA T
<u>Mitochondrial import receptor subunit Tom7 homolog</u>	Tom7	F CGT GCT GTA CCT CGG TTT CAA A R ACT CAA GAC CGT GGG CTC AG
<u>Mitochondrial import receptor subunit Tom5 homolog</u>	Tom5	F GGA GGA GAT GAA GAA GAA GAT GCG TCA AGA R CTC TGA GAA GGG CGA CGT AAA GAA GAA AGT
<u>Mitochondrial import inner membrane translocase subunit Tim44</u>	Tim44	F GAT GAC CTG GGA CAC ACT GG R TCA CTC CTC TTC CTG AGT CTG G
<u>Mitochondrial import inner membrane translocase subunit 23</u>	Tim23	F CAA GTC AGG AAG TGG CGT AA R AGA GCG TAG GCA CCA GAT A
<u>Mitochondrial import inner membrane translocase subunit Tim17-A</u>	Tim17A	F GGC GGT ATC CTT CTG GCA TTG R GGG AAC TGT GAG GAG GCA AAC
<u>Mitochondrial import inner membrane translocase subunit 16</u>	Tim16	F CGT GCC TTT GCT CGT GCC TTA R GCC TTC GCT GCT GCT TGA CT
<u>Mitochondrial import inner membrane translocase subunit 14</u>	Tim14	F AAT GAT CCT GAA CCA TCC AGA CAG AGG R GCC GTC CAT CAA ATC CTT CGC TTC
<u>Mitochondrial import inner membrane translocase subunit 13</u>	Tim13	F GGT TCG GTT CAG ACT TCT CA R GAC CTT GAC CTG CTC CAT
<u>Mitochondrial import inner membrane translocase subunit Tim8 A</u>	Tim8A	F CGA CAC CAC CCT GAC CAT CAC R CGC CCT TCT GCA CCA TCT GT

921

922

Table S2. Continued II.

Gene name	Symbol	Primer sequence
Mitochondrial import inner membrane translocase subunit Tim22	Tim22	F TCC GAC AGC ACG AGA AGT R AGA ACA TGG CAC CGA CGA T
Translocase of inner mitochondrial membrane 10 homolog	Tim10	F TAC CGC CAC ATT ACA AGG AGC R ATC CAG GCA CAC CGA CTC
Mitochondrial import inner membrane translocase subunit Tim9	Tim9	F CGT CAA AGA TTT CAC CAC CAG AGA G R GGA GAC ACG ACT CGG AGC A
Mitochondrial fission 1 protein	FIS1	F TCT CAG GAA CGA GCC AGG GAA CA R CCT TGT CGA TGA GTT TCT CCA GGT CCA G
Mitochondrial fission factor homolog B	MIFFB	F CGC AGC AGC ATT CCC TTC R CTC GTA CTG GAT TCG GTT CAT CT
Mitofusin 1	MFN1	F CAT CGT TGG AGG AGT GGT GTA R CCG TAC AGT GAG GCT GAG AG
Mitofusin 2	MFN2	F GGG ATG CCT CAG CCT CAG AAC CT R CTG CCT GCG GAC CTC TTC CAT GTA TT
Mitochondrial Rho GTPase 1	MIRO1A	F CAG GAC TTC TGC CGT AAG C R TAA GTG CAT CGG TCG TGT TG
Mitochondrial Rho GTPase 2	MIRO2	F TGA GGT GGA TGT GGA GGT GGA GTT R CAA GCA ACA TCA CAG GAG GCG TCT
Apoptosis-related protein 1	AIFM1	F ACA GAG GAG TCA GGA ACC R GGA GCA GGC AAT GAA GAG
Apoptosis-related protein 3	AIFM3	F GCA GCG GTA CAG TCT TGA ATG G R CCA GCG GAC GAG GAG CAA
Apoptosis regulator BAX	BAX	F GTG GCA GAC GGT GGG TGT TT R GCG AAT GAC GAG AAC AGT GGT GAG
Apoptosis regulator Bcl-2	BCL2	F GCT GTA TCT CAC CTC CAC CAC GG R TCT ATC ACC TCG GCG AAC CTC CT
Bcl-2-like protein 1	BCLX	F CGA CAT CAC TCC TGA CAC AGC CTA C R CCG TCC TTG AAC ACC TCG TCC ATC
p53 apoptosis effector related to PMP-22	PERP	F GGA GCA ACC ATC CTC AGC AT R GCG AGG CAG ACA GCA GAA
Transcription factor A, mitochondrial	mtTFA	F GAG CCC GCA ACA GAA ACA GCC ATT R ACT GCT CCC TGT CCC GCT GAT AG
Nuclear respiratory factor 1	NRF1	F CAG ATA GTC CTG GCA GAG A R GAC CTG TGG CAT CTT GAA
GA-binding protein alpha chain	NRF2	F CAT TGC CGT GGA CCG ATC TG R GCG TGT GAC CTG CTC TGA C
Proliferator-activated receptor gamma coactivator 1 alpha	PGC1 α	F CGT GGG ACA GGT GTA ACC AGG ACT C R ACC AAC CAA GGC AGC ACA CTC TAA TTC T
Proliferator-activated receptor gamma coactivator 1 beta	PGC1 β	F TCA GAG GAA GAG GCG GAT R GAC ACA GGT GGA GGA TGG

Table S3. Effect of three challenging stressors on liver mRNA gene expression. CTRL, control group; T-ST, thermal stress group; C-ST, chasing stress group; M-ST, multiple sensory perception stress group. Values are the mean $\pm$ SEM (n = 6-8). Rows with unlike superscript letters were significantly different (P<0.05; Student-Newman-Keuls). All data values were in reference to the expression level of proliferator-activated receptor gamma coactivator 1 β (PGC1 β) in CTRL fish, which was arbitrarily assigned a value of 1

Gene name*	CTRL	T-ST	C-ST	M-ST
mtHsp10	1.91 $\pm$ 0.33 ^a	3.54 $\pm$ 0.59 ^b	2.29 $\pm$ 0.38 ^{ab}	1.53 $\pm$ 0.14 ^a
DnaJA3A	0.35 $\pm$ 0.02	0.36 $\pm$ 0.06	0.38 $\pm$ 0.07	0.33 $\pm$ 0.05
DnaJC20	0.26 $\pm$ 0.02	0.29 $\pm$ 0.03	0.22 $\pm$ 0.02	0.23 $\pm$ 0.01
mtHsp60	0.75 $\pm$ 0.08 ^a	1.47 $\pm$ 0.23 ^b	0.64 $\pm$ 0.11 ^a	0.59 ^a $\pm$ 0.09
mtHsp70	1.22 $\pm$ 0.15	1.72 $\pm$ 0.16	1.19 $\pm$ 0.24	1.06 $\pm$ 0.18
DER-1	2.93 $\pm$ 0.20	3.96 $\pm$ 0.32	3.32 $\pm$ 0.38	2.57 $\pm$ 0.20
ERdj3	1.20 $\pm$ 0.20	1.52 $\pm$ 0.24	1.38 $\pm$ 0.27	0.93 $\pm$ 0.25
Grp-170	3.98 $\pm$ 0.45	4.31 $\pm$ 0.58	4.35 $\pm$ 0.59	2.83 $\pm$ 0.47
CAT	44.71 $\pm$ 5.80	54.80 $\pm$ 2.97	44.43 $\pm$ 5.36	47.64 $\pm$ 7.96
GPX4	24.14 $\pm$ 3.73	29.81 $\pm$ 1.57	22.78 $\pm$ 2.55	21.95 $\pm$ 2.27
GR	0.69 $\pm$ 0.04	0.84 $\pm$ 0.04	0.67 $\pm$ 0.07	0.70 $\pm$ 0.14
GST3	9.80 $\pm$ 1.78	11.56 $\pm$ 0.79	9.15 $\pm$ 1.56	13.75 $\pm$ 2.84
PRDX3	1.63 $\pm$ 0.20	1.72 $\pm$ 0.21	1.69 $\pm$ 0.17	1.42 $\pm$ 0.28
PRDX5	1.81 $\pm$ 0.19	2.06 $\pm$ 0.18	1.96 $\pm$ 0.20	2.06 $\pm$ 0.37
SOD2	3.12 $\pm$ 0.41	2.41 $\pm$ 0.30	2.58 $\pm$ 0.26	2.86 $\pm$ 0.44
ACAA2	0.55 $\pm$ 0.07	0.53 $\pm$ 0.08	0.44 $\pm$ 0.08	0.36 $\pm$ 0.03
CPT1A	0.94 $\pm$ 0.13 ^b	3.75 $\pm$ 0.26 ^c	0.86 $\pm$ 0.21 ^b	0.49 $\pm$ 0.12 ^a
ECH	4.23 $\pm$ 0.19	4.77 $\pm$ 0.63	4.72 $\pm$ 0.58	4.01 $\pm$ 0.61
HADH	5.24 $\pm$ 0.44	6.81 $\pm$ 0.59	4.76 $\pm$ 0.42	4.15 $\pm$ 0.97
CS	2.35 $\pm$ 0.09 ^b	4.26 $\pm$ 0.32 ^c	2.11 $\pm$ 0.23 ^b	1.49 $\pm$ 0.12 ^a
IDH3A	0.02 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.02 $\pm$ 0.00
IDH3B	0.24 $\pm$ 0.03	0.29 $\pm$ 0.03	0.23 $\pm$ 0.03	0.23 $\pm$ 0.04
IDH3G	0.38 $\pm$ 0.01	0.40 $\pm$ 0.04	0.30 $\pm$ 0.04	0.33 $\pm$ 0.04
Cox4a	0.13 $\pm$ 0.01	0.17 $\pm$ 0.01	0.12 $\pm$ 0.01	0.14 $\pm$ 0.01
UCP1	18.07 $\pm$ 4.17	20.71 $\pm$ 1.85	17.86 $\pm$ 4.30	18.78 $\pm$ 3.35
UCP2	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00
UCP3	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Tom70	1.53 $\pm$ 0.11 ^a	2.47 $\pm$ 0.17 ^b	1.46 $\pm$ 0.11 ^a	1.49 $\pm$ 0.22 ^a
Tom34	0.45 $\pm$ 0.03 ^a	0.64 $\pm$ 0.05 ^b	0.46 $\pm$ 0.06 ^a	0.38 $\pm$ 0.03 ^a
Tom22	0.57 $\pm$ 0.04 ^a	1.20 $\pm$ 0.17 ^b	0.74 $\pm$ 0.12 ^a	0.82 $\pm$ 0.07 ^a
Tom7	0.88 $\pm$ 0.12	0.83 $\pm$ 0.09	0.66 $\pm$ 0.03	0.69 $\pm$ 0.12
Tom5	0.78 $\pm$ 0.09	0.85 $\pm$ 0.07	0.87 $\pm$ 0.08	0.88 $\pm$ 0.19

Gene name*	CTRL	T-ST	C-ST	M-ST
Tim44	0.08 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.07 ± 0.01
Tim23	0.38 ± 0.02	0.53 ± 0.05	0.48 ± 0.08	0.38 ± 0.05
Tim17A	1.41 ± 0.14	1.56 ± 0.09	1.48 ± 0.14	1.22 ± 0.17
Tim16	0.31 ± 0.02	0.32 ± 0.02	0.27 ± 0.02	0.26 ± 0.04
Tim14	1.72 ± 0.21	1.73 ± 0.06	1.51 ± 0.11	1.49 ± 0.11
Tim13	0.39 ± 0.03	0.48 ± 0.07	0.34 ± 0.06	0.34 ± 0.05
Tim8A	1.06 ± 0.06	1.09 ± 0.15	0.91 ± 0.09	0.78 ± 0.08
Tim22	0.22 ± 0.03	0.27 ± 0.03	0.22 ± 0.05	0.20 ± 0.04
Tim10	0.41 ± 0.08 ^a	0.70 ± 0.05 ^b	0.38 ± 0.10 ^a	0.39 ± 0.08 ^a
Tim9	0.37 ± 0.04 ^a	0.60 ± 0.06 ^b	0.36 ± 0.06 ^a	0.31 ± 0.03 ^a
FIS1	2.11 ± 0.15	2.79 ± 0.18	2.11 ± 0.15	1.91 ± 0.16
MIFFB	0.25 ± 0.02 ^b	0.23 ± 0.02 ^{ab}	0.17 ± 0.01 ^a	0.18 ± 0.03 ^a
MFN1	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
MFN2	0.44 ± 0.03 ^a	0.62 ± 0.07 ^b	0.38 ± 0.04 ^a	0.30 ± 0.04 ^a
MIRO1A	0.11 ± 0.01	0.13 ± 0.01	0.11 ± 0.02	0.10 ± 0.02
MIRO2	0.71 ± 0.03	0.74 ± 0.04	0.77 ± 0.10	0.59 ± 0.04
AIFM1	2.62 ± 0.39	3.26 ± 0.55	2.28 ± 0.19	3.01 ± 0.98
AIFM3	0.41 ± 0.03 ^{ab}	0.50 ± 0.04 ^b	0.36 ± 0.02 ^{ab}	0.32 ± 0.06 ^a
BAX	0.30 ± 0.03 ^b	0.35 ± 0.03 ^b	0.26 ± 0.02 ^{ab}	0.19 ± 0.02 ^a
Bcl-2	0.44 ± 0.08	0.38 ± 0.01	0.34 ± 0.03	0.34 ± 0.04
BCLX	0.81 ± 0.05 ^b	0.88 ± 0.08 ^b	0.67 ± 0.05 ^{ab}	0.55 ± 0.04 ^a
PERP	2.30 ± 0.15	2.35 ± 0.11	2.31 ± 0.17	1.97 ± 0.13
mtTFA	0.43 ± 0.03	0.37 ± 0.04	0.38 ± 0.04	0.36 ± 0.02
NRF1	0.14 ± 0.01 ^a	0.33 ± 0.04 ^b	0.13 ± 0.01 ^a	0.10 ± 0.01 ^a
NRF2	0.28 ± 0.02 ^a	0.51 ± 0.04 ^b	0.24 ± 0.01 ^a	0.20 ± 0.01 ^a
PGC1α	0.05 ± 0.00 ^b	0.32 ± 0.06 ^c	0.07 ± 0.02 ^{ab}	0.02 ± 0.01 ^a
PGC1β	1.05 ± 0.11	1.05 ± 0.13	1.21 ± 0.43	1.23 ± 0.16

933 * Gene identity determined through BLAST searches: mtHsp10, 10 kDa heat shock protein,
934 mitochondrial; DnaJA3Aa, 40 kDa heat shock protein DnaJ (Hsp40) homolog, subfamily A, member 3A;
935 DnaJC20, Iron-sulfur cluster co-chaperone protein HscB; mtHsp60, 60 kDa heat shock protein,
936 mitochondrial; mtHsp70, 70 kDa heat shock preotin, mitochondrial; DER-1, Derlin-1; ERdj3, ER-
937 associated Hsp40 co-chaperone; Grp-170, 170 kDa Glucose-regulated protein; CAT, Catalase; GPX4,
938 Glutathione peroxidase 4; GR, Glutathione reductase; GST3, Glutathione S-transferase 3; PRDX3,
939 Peroxiredoxin 3; PRDX5, Peroxiredoxin 5; SOD2, Superoxide dismutase [Mn]; ACAA2, 3-ketoacyl-CoA
940 thiolase, mitochondrial; CPT1A, Carnitine palmitoyltransferase 1A; ECH, Enoyl-CoA hydratase,
941 mitochondrial; HADH, Hydroxyacyl-CoA dehydrogenase; CS, Citrate synthase; IDH3A, Isocitrate
942 dehydrogenase [NAD] subunit alpha, mitochondrial; IDH3B, Isocitrate dehydrogenase [NAD] subunit
943 beta, mitochondrial; IDH3G, Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial; Cox4a,
944 Cytochrome C oxidase subunit IV isoform 1; UCP1, Uncoupling protein 1, UCP2, Uncoupling protein 2;
945 UCP3, Uncoupling protein 3; Tom70, Mitochondrial import receptor subunit Tom70; Tom34,
946 Mitochondrial import receptor subunit Tom34; Tom22, Mitochondrial import receptor subunit Tom22;
947 Tom7, Mitochondrial import receptor subunit Tom7 homolog; Tom5, Mitochondrial import receptor
948 subunit Tom5 homolog; Tim44, Mitochondrial import inner membrane translocase subunit Tim44;
949 Tim23, Mitochondrial import inner membrane translocase subunit 23; Tim17A, Mitochondrial import
950 inner membrane translocase subunit Tim17-A; Tim16, Mitochondrial import inner membrane translocase
951 subunit 16; Tim14, Mitochondrial import inner membrane translocase subunit 14; Tim13, Mitochondrial
952 import inner membrane translocase subunit 13; Tim8A, Mitochondrial import inner membrane

953 translocase subunit Tim8 A; Tim22, Mitochondrial import inner membrane translocase subunit Tim22;
 954 Tim10, Translocase of inner mitochondrial membrane 10 homolog; Tim9, Mitochondrial import inner
 955 membrane translocase subunit Tim9; FIS1, Mitochondrial fission 1 protein; MIFFB, Mitochondrial
 956 fission factor homolog B; MFN1, Mitofusin 1; MFN2, Mitofusin 2; MIRO1A, Mitochondrial Rho
 957 GTPase 1; MIRO2, Mitochondrial Rho GTPase 2; AIFM1, Apoptosis-related protein 1; AIFM3,
 958 Apoptosis-related protein 3; BAX, Apoptosis regulator BAX; BCL2, Apoptosis regulator Bcl-2; BCLX,
 959 Bcl-2-like protein 1; PERP, p53 apoptosis effector related to PMP-22; mtTFA, Transcription factor A,
 960 mitochondrial; NRF1, Nuclear respiratory factor 1; NRF2, Nuclear respiratory factor 2; PGC1 α ,
 961 Proliferator-activated receptor gamma coactivator 1 alpha; PGC1 β , Proliferator-activated receptor gamma
 962 coactivator 1 beta.